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Modulation of bradykinin-induced intracellular Ca^{2+} oscillations in v-Ki-*ras*-transformed fibroblasts[1]

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ABSTRACT Modulation of bradykinin (BK)-induced intracellular Ca^{2+} oscillations was investigated with single cell Ca^{2+} anlysis in v-Ki-*ras*-transformed NIH3T3 fibroblasts. The Ca^{2+} oscillations were inhibited by the addition of a specific antagonist for subtype 2 of BK receptors (B_2 receptor), not the antagonist for B_1 receptor. Decrease of the extracellular Ca^{2+} concentration suppressed the $[Ca^{2+}]_i$ oscillations and application of thapsigargin dissipated the $[Ca^{2+}]_i$ oscillations. These findings suggest that the continuous activation of B_2 receptor leading to the fluctuations of both Ca^{2+} influx which refills the internal Ca^{2+} stores, and Ca^{2+} mobilization from the internal stores, is essential to the occurrence of the $[Ca^{2+}]_i$ oscillations in these cells.

KEY WORDS bradykinin; calcium; oncogenes; fibroblasts

Bradykinin (BK), the mitogenic peptide in fibroblasts[1], induced $[Ca^{2+}]_i$ oscillations in NIH3T3 fibroblasts expressed v-Ha-*ras* gene or v-Ki-*ras* gene, but not in their parent cells, and the $[Ca^{2+}]_i$ oscillations were strongly correlated with the malignant properties, the membrane potential changes and the alterations in phospholipid metabolism[2,3]. BK and its analogs exert their effects by interacting with at least 2 different subtypes of receptors and the multiple signal transduction pathways, which may affect the intracellular Ca^{2+} homeostasis, are involved in BK stimulations[4,5]. In the present study, the effects of B_1 and B_2 antagonists on intracellular calcium responses triggered by BK in v-Ki-*ras*-transformed fibroblasts were investigated, and the dependence of Ca^{2+} oscillations on either extracellular Ca^{2+} or intracellular Ca^{2+} storages was evaluated.

MATERIALS AND METHODS

Materials BK, Arg[0][Hyp[3], Thi[5,8], D-Phe[7]]BK, des-Arg[9][Leu[8]]BK, Fura 2-AM, and thapsigargin were obtained from Sigma (St Louis MO, USA). The media and sera for cell culture were from Gibco (Grand Island NY, USA). All other chemicals were from commercial sources.

Cell culture v-Ki-*ras*-Transformed NIH3T3 fibroblasts were grown in Dulbecco's modified Eagle medium supplemented with 10 % fetal calf serum[3]. Special care was taken to maintain the cell line at a subconfluent density, and cultures were replaced at 1-month intervals from frozen stocks.

Measurement of intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ was determined by fluorescent Ca^{2+} indicator Fura 2[6] with a modified method[3]. Cells were plated at a density of 5×10^5 cells/chamber on a glass coverslip attached to Flexipern-Disc and precoated with gelatin. After being cultured at 37 C for 48 h, the cells were kept in the serum-free medium for 24 h and then labeled with Fura 2-AM (2.5 $\mu mol\cdot L^{-1}$) at 37 C for 30 min in 0.5 ml of the assay buffer (NaCl 125, HEPES 25, KCl 5, $CaCl_2$ 1, $MgCl_2$ 1, glucose 10 $mmol\cdot L^{-1}$ and 0.1 % bovine serum albumin, pH 7.05). Fluorescence images were obtained with dual

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excitation wavelengths set at 340 nm/360 nm and emission wavelength at 500 nm. Band passes were 10 nm for λ_{ex} and 20 nm for λ_{em}. The intracellular calcium concentrations were calculated[6] and evaluated with t test.

RESULTS AND DISCUSSION

Profile of BK-induced $[Ca^{2+}]_i$ oscillations in v-Ki-*ras*-transformed fibroblasts The amplitude and frequency of BK-induced $[Ca^{2+}]_i$ oscillations in v-Ki-*ras*-transformed fibroblasts loaded with Fura-2 exhibited a distinct concentration-dependent manner. BK 1 — 100 nmol·L^{-1} increased the amplitude of $[Ca^{2+}]_i$ spikes and decreased the frequency. The frequency of the oscillations was inversely correlated to the amplitude (Tab 1).

Tab 1. Effects of BK on $[Ca^{2+}]_i$ in v-Ki-*ras*-transformed NIH3T3 fibroblasts, $n=21$, $\bar{x}\pm s$. Unstimulated level of $[Ca^{2+}]_i$ in cells was 0.12±0.03 μmol·L^{-1}). $^cP<0.01$ *vs* BK 1 nmol·L^{-1}.

BK/ nmol·L^{-1}	$[Ca^{2+}]_i$ spikes	
	Amplitude/ μmol·L^{-1}	Frequency/ cycle·min^{-1}
1	0.33±0.06	0.47±0.03
10	0.48±0.05^c	0.41±0.02^c
100	0.83±0.17^c	0.27±0.03^c

The $[Ca^{2+}]_i$ oscillations induced by BK were inhibited by pretreatment with SK&F96365, a receptor-operated Ca^{2+} channel blocker[7], as well as the inorganic Ca^{2+} channel blockers, such as Ni^{2+} and Cd^{2+}, but not nifedipine, a dihydropyridine antagonist (data not shown). Removal of BK terminated the $[Ca^{2+}]_i$ oscillations already in progress, although the stoppage was never instaneous as the cells normally gave 1 and occassionally 2 more $[Ca^{2+}]_i$ spikes before ceasing to oscillate altogether. These observations suggest that the receptor-mediated Ca^{2+} entry is involved in the $[Ca^{2+}]_i$ oscillations.

Effects of B_1 and B_2 antagonists on BK-induced $[Ca^{2+}]_i$ oscillations B_1 antagonist des-Arg^9[Leu^8]BK (0.1 — 10 μmol·L^{-1}) did not inhibit the BK-induced $[Ca^{2+}]_i$ oscillations in the cells. B_2 antagonist Arg^0[Hyp^3, Thi^{5,8}, D-Phe^7]BK (10 μmol·L^{-1} either before or after the addition of BK (100 nmol·L^{-1}) abolished the oscillations (Fig 1). These findings indicate that BK - induced $[Ca^{2+}]_i$ oscillations are due to the activation of B_2 receptor.

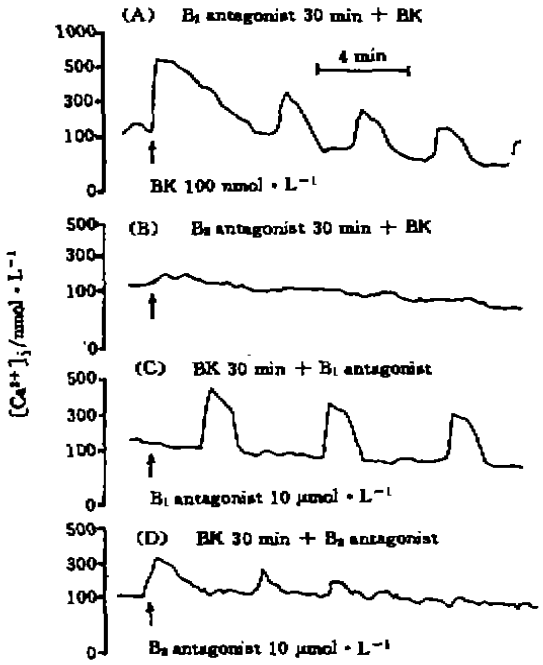

Fig 1. Effects of B_1 antagonist des-Arg^9[Leu^8]BK and B_2 antagonist Arg^0[Hyp^3, Thi^{5,8}, D-Phe^7]BK on BK-induced $[Ca^{2+}]_i$ oscillations. The cells were stimulated with BK after pretreatment with either B_1 antagonist (A) or B_2 antagonist (B) 10 μmol·L^{-1} for 30 min. B_1 antagonist (C) or B_2 antagonist (D) was added after BK 100 nmol·L^{-1} for 30 min. Results shown were obtained from at least 3 separate experiments and >30 cells.

In fibroblasts, activation of B_2 receptor is considered to be functionally linked to the release of eicosanoids and cAMP as well as to

the formation of inositol-1,4,5-trisphosphate ($IP_3(1,4,5)$) mediated by guanine nucleotide-binding proteins (G-proteins)[4,8]. On the other hand, B_2 receptor undergoes ligand-induced down-regulation or desensitization due either to a reduction of the receptor numbers on the cell surface by internalization or to a decrease of receptor affinity in fibroblasts. Interestingly, in several fibroblast cell lines transfected with *ras*-oncogenes, the number of BK receptor is elevated as compared with those in their parent cells[9,10]. In the v-Ki-*ras*-transformed fibroblasts, high-affinity [^{3}H]BK binding (for B_2 receptor) is increased and phosphoinositides responses are enhanced as compared with that in their parent NIH3T3 cells[10]. These raise the possibility that Ras proteins can increase the BK receptor expression. The alternative possibility is that Ras proteins attenuate the down-regulation or the desensitization of BK receptor during homologous or heterologous stimulation.

Contribution of extracellular Ca^{2+} and internal stores of Ca^{2+} to the $[Ca^{2+}]_i$ oscillations induced by BK stimulation BK-induced $[Ca^{2+}]_i$ oscillations were dependent on the presence of extracellular Ca^{2+} (Fig 2). In Ca^{2+}-free assay buffer, BK only produced a transient $[Ca^{2+}]_i$ response. The $[Ca^{2+}]_i$ oscillations occurred with the addition of Ca^{2+} 0.5 mmol·L^{-1} to assay buffer (Fig 2A). The amplitude of $[Ca^{2+}]_i$ spikes was increased when the extracellular Ca^{2+} concentration was elevated from 0.5 to 2 mmol·L^{-1}. While the extracellular Ca^{2+} concentration was drastically decreased from 2 to 0.15 μmol·L^{-1} by the application of excess egtazic acid (5 mmol·L^{-1}), the $[Ca^{2+}]_i$ oscillations were abolished (Fig 2B).

On the other hand, a type of distinct Ca^{2+} current oscillations induced by BK has been found in these cells, while not in their parent

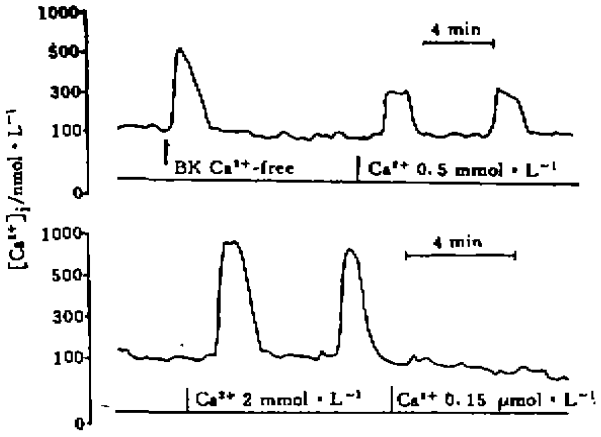

Fig 2. Effect of extracellular Ca^{2+} on BK-induced $[Ca^{2+}]_i$ oscillations. (A) BK 100 nmol·L^{-1} was added into Ca^{2+}-free buffer. After 12 min of the BK stimulation, extracellulr Ca^{2+} concentration was increased to 0.5 mmol·L^{-1}. (B) extracellular Ca^{2+} concentration was first increased from 0.5 to 2 mmol·L^{-1}, then decreased to 0.15 μmol·L^{-1}.

NIH3T3 cell[11]. Similar to $[Ca^{2+}]_i$ oscillations, the Ca^{2+} current oscillations were inhibited by pretreatment with SK&F96365, as well as Ni^{2+} or Cd^{2+}. Taken together with above data, it raises the question if the $[Ca^{2+}]_i$ oscillations detected in Fura-2 loaded cells represent the Ca^{2+} current oscillations which could be detected by the patch clamp method.

The Ca^{2+} current oscillations recorded by a patch clamp in these cells also showed that the frequency of the Ca^{2+} current oscillations was inversely correlated with amplitude. However, at the same dose of BK, the Ca^{2+} current oscillations appear with much higher frequency (2.4 to 23.6-fold) than that of $[Ca^{2+}]_i$ oscillations. This may imply that Ca^{2+} current oscillations, which produce the Ca^{2+} inflow waves, do not directly elicit the detectable increase of cytosolic free Ca^{2+}, but refill the internal Ca^{2+} stores. That is, Ca^{2+} would enter the intracellular dischargeable

stores directly from the external medium through the receptor-operated Ca^{2+} channel, or following the Ca^{2+} influx, the elevated intracellular free Ca^{2+}, may be pumped rapidly into the internal stores.

In order to test the above hypothesis, we investigated the contribution of internal Ca^{2+} stores to the $[Ca^{2+}]_i$ oscillations by using thapsigargin (TG), an inhibitor of microsomal Ca^{2+}-ATPase[11,23]. This tumor-promoting sesquiterpene lactone depletes Ca^{2+} from the intracellular stores without concomitant release of inositol phosphates in many types of cells, such as platelets, hepatocytes, and neuroblastoma cells[12]. In the v-Ki-*ras*-transformed cells, TG (200 nmol·L^{-1}) did induce large $[Ca^{2+}]_i$ spike, even in the absence of extracellular Ca^{2+} (Fig 3A and 3B), probably reflecting the inhibition of Ca^{2+} uptake via Ca^{2+}-ATPase on surface membrane of Ca^{2+} pool and the depletion of Ca^{2+} from the pool. Pretreatment of the cells with TG (200 nmol·L^{-1}) for 30 min, completely abolished BK — induced $[Ca^{2+}]_i$ responses (Fig 3C). When TG (200 nmol·L^{-1}) was added 30 min after application of BK, the $[Ca^{2+}]_i$ oscillations were terminated (data not shown).

There are several hypotheses to explain the mechanisms of $[Ca^{2+}]_i$ oscillations in nonexcitable cells[13]. In the v-Ki-*ras*-transformed fibroblasts, it is possible that Ras protein increases the BK receptor, especially B_2 receptor number. Thus, the signal transduction of BK is enhanced. Although the possibility of the contribution of other signal transducing pathways in the $[Ca^{2+}]_i$ oscillations can not be excluded, the enhanced phosphoinositide turnover (PI turnover), which generates two second messenger $IP_3(1,4,5)$ and diacylglycerol, is a good candidate. In the v-Ki-*ras*-transformed cells, BK did induce a much higher level of $IP_3(1,4,5)$ generation as compared with that in their parent NIH3T3 cells[17]. Taken together with the observation of effects of TG on the $[Ca^{2+}]_i$ oscillations, the evidence supports the notion that the PI turnover could serve as an oscillator.

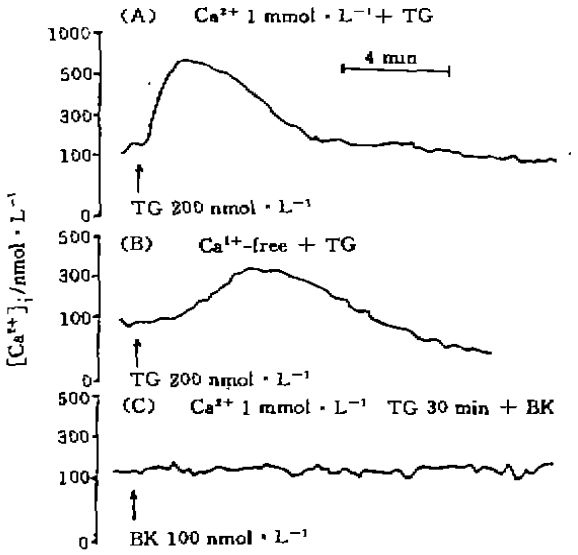

Fig 3. Effects of TG on $[Ca^{2+}]_i$ in cells. TG was added into assay buffer containing Ca^{2+} 1 mmol·L^{-1} (A), or Ca^{2+}-free buffer containing egtazic acid 1 mmol·L^{-1} (B). After 30 min in the same buffer containing TG as that in Fig 3A, BK was added (C).

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缓激肽受体介导的 v-Ki-*ras* 转化成纤维细胞内钙离子振荡的调节[1]

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摘要 由单个细胞内钙离子的分析显示: 缓激肽 B_2 受体拮抗剂, 抑制了缓激肽所诱导的在 v-Ki-*ras* 转化成纤维细胞内的钙离子振荡, B_1 受体拮抗剂无此作用. 降低胞外钙离子浓度以及用 thapsigargin 处理细胞均阻止了钙离子振荡的发生. 这些结果提示, 持续地激活 B_2 受体, 导致钙离子内流及钙离子从内源性贮存部位释放的波动, 从而引起钙离子的振荡.

关键词 缓激肽; 钙; 癌基因; 成纤维细胞

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Enhancement of interleukin-2 production and its mRNA expression by dihydroartemisinin[1]

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ABSTRACT Immunoregulatory properties of a novel antimalarial drug dihydroartemisinin (DHA) were investigated *in vitro*. DHA 0.5—5 $\mu mol \cdot L^{-1}$ enhanced the lymphocyte proliferation induced by Con A. Interleukin 2 (IL-2) production and its mRNA expression by both Con A-stimulated mouse splenocytes and a T cell line LBRM-33-1A5 were also augmented by DHA. In contrast, DHA 0.5—5 $\mu mol \cdot L^{-1}$ did not show any effect on the lipopolysaccharides (LPS)-induced lymphocyte proliferation and the spontaneous and mitogen-induced proliferation of transformed T cells. These results indicated that DHA might regulate lymphocyte responses through

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